Effects of Leptin Deficiency and Short-Term Repletion on Hepatic Gene Expression in Genetically Obese Mice

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By supplying most organs of the body with metabolic substrates, the liver plays a central role in maintaining energy balance. Hepatic metabolism of glucose, fatty acids, and lipoproteins is disrupted in the leptin-deficient obese (*Lep^{ob}/Lep^{ob}*) mouse, leading to hyperglycemia, steatosis, and hypercholesterolemia. Microarray expression profiles were used to identify transcriptional perturbations that underlie the altered hepatic physiology of *Lep^{ob}/Lep^{ob}* mice. A wide variety of genes involved in fatty acid metabolism are altered in expression, which suggests that both fatty acid synthesis and oxidation programs are activated in obese mice. The expression of a small subset of genes is upregulated by leptin deficiency, not modulated by caloric restriction, and markedly suppressed by short-term leptin treatment. Among these leptin-regulated genes, apolipoprotein A-IV is a strong candidate for mediating the atherogenic-resistant phenotype of *Lep^{ob}/Lep^{ob}* mice.
***Diabetes* 50:2268–2278, 2001**

Mammals maintain energy homeostasis by an incompletely understood network of neuroregulatory and metabolic pathways. These systems provide all tissues with a steady and sufficient supply of energy in the face of fluctuations in food intake and metabolic needs. In humans, the normal control of homeostatic processes, including energy balance, likely requires complex interactions among hundreds of genes and gene products in multiple organ systems (1). During the past decade, the molecular characterization of single-gene obesity syndromes in rodents (obese, diabetes, lethal yellow, tubby, and fat in mice and fatty in rats) has allowed identification of a few important regulators of mammalian energy homeostatic systems, but the regulatory map remains incomplete (2).

Two of these loci, obese (*Lep^{ob}/Lep^{ob}*) and diabetes (*Lepr^{db}/Lepr^{db}*), harbor mutations in the leptin (LEP) and leptin receptor (LEPR) genes, respectively (3–6). Leptin, a hormone secreted primarily by adipocytes in proportion to adipose mass, serves as a measure of total body energy stores. LEPR is widely expressed in at least five isoforms, but the LEPR-B isoform seems to mediate most, if not all, of leptin's energy-regulating effects. Consistent with this hypothesis, the LEPR-B isoform is specifically truncated in the original *Lepr^{db}* allele (4–6) and is most highly expressed within energy-regulating regions of the hypothalamus, including the arcuate, paraventricular, dorsomedial, ventromedial, and lateral nuclei (7,8). In the absence of leptin or a functional LEPR-B, rodents and humans develop a complex metabolic and behavioral syndrome of hyperphagia, hypogonadotropism, decreased energy expenditure, severe insulin resistance, and morbid obesity (9,10). Molecular characterization of leptin and LEPR have allowed identification of an important pathway in the mammalian energy homeostatic system.

Although rare humans and animal strains with mutations in single genes have provided key insights, a more complete understanding of mammalian energy homeostasis and its derangement in diabetes and obesity will require methods and analytical tools that can measure the actions and interactions of thousands of genes simultaneously. Microarrays can be used to monitor the variation and expression of tens of thousands of genes, measuring on a global scale differences between normal and pathologic states (11,12). Early studies using microarrays compared patterns of gene expression among various benign and neoplastic hematopoietic cells, normal and failing hearts (13), and, recently, adipose tissue from lean and genetically obese mice (14). These studies have been useful in identifying patterns of expression that are specific for pathologic states and, in the case of hematopoietic malignancies, identifying clinically important subclasses of lymphomas and leukemias (15,16).

Here we report the production of spotted microarrays and an assessment of their specificity in detecting differences in gene expression and their use to generate expression profiles from livers of *Lep^{ob}/Lep^{ob}* and wild-type mice. The liver serves as a primary center for gluconeogenesis, fatty acid metabolism, ketone body production, and lipoprotein trafficking and, therefore, is a key regulator of total body energy homeostasis. In *Lep^{ob}/Lep^{ob}* mice, each of these processes has been characterized and found to be perturbed, contributing to the overall leptin-deficient phenotype. These expression data identify sets of genes whose expression is altered in the absence of leptin and corrected by short-term caloric restriction or leptin treatment. These genes represent candidates for mediating specific aspects of the obese phenotype and the leptin-specific component of mammalian energy homeostatic pathways.

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Received for publication 2 April 2001 and accepted in revised form 19 June 2001.

Apo A-IV, apolipoprotein A-IV; dNTP, deoxynucleotide triphosphates; dUTP; deoxyuridine-triphosphate; HMG, hydroxymethylglutaryl; IIP2, insulin-induced protein 2; PCR, polymerase chain reaction; SSC, sodium chloride-sodium citrate; SREBP-1, sterol regulatory element-binding protein 1.

RESEARCH DESIGN AND METHODS

Preparation of polymerase chain reaction products and glass substrate slides. Libraries of Unigene murine cDNAs were obtained from Research Genetics (Huntsville, AL) as bacterial colonies in a 96-well microtiter plate format. Plasmids that represented each clone were isolated with the use of a high-throughput purification system (Turbo Miniprep; Qiagen, Valencia, CA). The insert from each clone was amplified in a 75-μl polymerase chain reaction (PCR), using vector-derived primers (T7–5′-AATTAACCCTCACTAAAGGG-3′ and T3–5′-TAATACGACTCACTATAGGG-3′). To ensure that successful and specific amplification was achieved, 3 μl of each product was electrophoresed on a 1% agarose/Tris-borate-EDTA gel. The remainder (~72 μl) of reaction mixes that yielded single products of the appropriate size and concentration were ethanol precipitated and resuspended in 3× sodium chloride–sodium citrate (SSC) with a final DNA concentration of 0.2–0.6 μg/μl. The DNA was transferred to new 96-well plates, sealed, and stored at −20 until arrayed. Polylysine-coated glass 75 × 25-mm microscope slides were obtained commercially (Sigma, St. Louis, MO).

Arraying PCR products. All arraying was performed with a four-pin-head GMS 417 Arrayer (Affymetrix, Santa Clara, CA) according to the manufacturer's instructions at a density of 1,300 spots/cm^2. The robotics systems are self-contained within a Plexiglas enclosure and maintained at a relative humidity of 40–50% and temperature of 23–26°C during arraying. The microarrays were stored in a low-humidity environment for >2 weeks before use.

Preprocessing of microarrays. For ensuring covalent linkage of PCR products to the polylysine surface, microarrays were preprocessed on the day of hybridization. Each microarray was moistened over a bath of heated water (95°C) and exposed to 60 mJ ultraviolet energy. After cross-linking, the microarrays were washed serially in 0.1% SDS, 0.2× SSC, and 100% ethanol and dried by centrifugation in a tabletop centrifuge adapted for this purpose. Dried microarrays were prehybridized immediately.

Generation of fluorescently labeled cDNA from polyA RNA. Total RNA from 50 mg liver was extracted with the use of an acid-phenol method according to the manufacturer's instructions (Trizol; BRL Gibco, Gaithersburg, MD). From each sample, 0.5–1.0 μg polyA RNA was isolated using a polydeoxythymidine resin (Oligotex; Qiagen). PolyA RNA was transcribed into fluorescently labeled cDNA using an anchored oligo-dT primer ($dT_{18}MN$), unlabeled deoxynucleotide triphosphates (dNTPs), reverse transcriptase, and deoxyuridine-triphosphate (dUTP) labeled with one of two fluorophores, Cy3 or Cy5 (17). Unincorporated dNTPs were removed, and the sample was concentrated with the use of a minifiltration system (YM-30; Microcon, Bedford, MA).

Hybridization and washing of microarrays. Microarrays were prehybridized with 15 μl of a formamide-based hybridization solution (0.75 mol/l NaCl, 0.15 mol/l Tris-HCl [pH 7.5], 0.1 mol/l Na_2HPO_4, 0.1% $Na_4HP_2O_7$, 2% SDS, 5× Denhardt's solution, 100 μg/ml sheared salmon sperm DNA, and 50% formamide) for 1 h at 42°C in a humidified hybridization chamber. For each microarray, Cy5- and Cy3-labeled cDNA from two separate mouse liver RNA extractions were combined with 10 μl hybridization buffer, heated at 95°C for 10 min, and centrifuged for 5 min in a microfuge at maximum speed to remove any particulate matter. The combined sample was placed on a prehybridized microarray and covered with a coverslip. The coverslip was held in place by rubber cement, and the microarray was hybridized for 12–14 h at 42°C in a humidified chamber. After hybridization, the coverslip was removed and the microarray was washed at room temperature in serial 3-min washes of 0.1× SSC/0.1% SDS and two washes of 0.1× SSC. The slides were dried by centrifugation (1,000*g*) in a tabletop centrifuge.

Fluorescent scanning of microarrays. The microarrays were scanned according to the manufacturer's instructions with the use of an Affymetrix 428 Array Scanner. The scan of each microarray generated two 16-bit TIFF image files, one representing the fluorescence emission from each fluorophore.

Extracting data from TIFF images. Paired image files, generated from scans of microarrays, were analyzed with the use of Imagene software (BioDiscovery, Los Angeles, CA). This software package identifies spots within an array image and calculates mean, total, and median signal intensity values for the pixels that constitute each spot. In addition, the software calculates local background intensities and allows for outlier pixels to be excluded from calculations. In combination with CloneTracker 2.0 (BioDiscovery), the Imagene software correlates each spot with a specific cDNA clone and generates a spreadsheet output file containing mean signal and local background for each spot.

Calculating expression ratios. After the raw data from a single microarray were extracted from two TIFF images, they were downloaded into a single spreadsheet. Subtracting the mean local background from the mean signal of each spot generated a net signal for each represented cDNA. When the net signal for any spot was both greater than the average background for the entire array and greater than the local background, that spot was used for subsequent calculations. When both of these criteria were not met, a spot and the cDNA that it represents were not likely to give reliable data and, therefore, were not considered in further calculations. The sum of the net signal for each spot on both images was calculated, and the ratio between the sums for each image was defined as the normalization factor. Individual normalized expression ratios were calculated by dividing the net signal of a given spot i from image A by the net signal from the same spot on image B, multiplied by the normalization factor:

$$\text{Mean normalized ratio for cDNA}_i = (\text{net signal A})_i/(\text{net signal B})_i \times (\Sigma \text{ net signal B})/(\Sigma \text{ net signal A})$$

Indentifying differentially regulated genes. Each microarray experiment was performed in triplicate using RNA extracted from a total of six animals. Thus, in comparing wild-type (*Lep*$^+$/*Lep*$^+$ C57BL/6J) with obese (*Lep*^{ob}/*Lep*^{ob} C57BL/6J) expression profiles, RNA was extracted separately from the three mice of each genotype to generate labeled cDNA. The labeled cDNA from a single wild-type mouse was combined with cDNA from a single obese mouse and hybridized to a microarray. Normalized mean ratio data were calculated for each cDNA on each microarray, and those sets of genes that showed a consistent 30% or greater increase or decrease in expression between two genotype/treatment groups were identified. The normalized mean ratio data were then used to calculate mean ratios for genes that demonstrated consistent changes.

General animal care. All mice were females purchased from Jackson Laboratory (Bar Harbor, ME) at 4–6 weeks of age and acclimatized for at least 1 week before experimental manipulation or killing. The mice were kept individually in Plexiglas, ventilated cages with cob bedding and except where noted had free access to water and a standard pellet diet, which derives 10% of calories from fat (PicoLab Diets). The temperature was kept at 22 ± 1°C, and a 12-h light-dark cycle was maintained. All animals were killed by CO_2 asphyxiation 1 h into the light cycle. Columbia's Institutional Animal Care and Use Committee guidelines were followed at all times.

Leptin injections. Recombinant murine leptin was purchased from Peprotech (Princeton, NJ) and resuspended according to the manufacturer's instructions in sterile phosphate-buffered saline, pH 7.4 (1 mg/ml), and sterile filtered (0.22-μm cellulose acetate filter). Resuspended leptin was stored at −20°C for no more than 2 weeks. Leptin-treated animals were weighed and received an intraperitoneal injection once daily 1 h before the dark cycle with 0.5 μg/g. Vehicle-treated animals were handled identically to the leptin-treated animals except that they received an injection of phosphate-buffered saline.

Northern blot analysis. Ten micrograms of total RNA was extracted from the livers of *Lep*^{ob}/*Lep*^{ob} or wild-type C57BL/6J mice, electrophoresed through denaturing formaldehyde agarose gels, blotted to nylon membranes, and probed with ^{32}P-labeled, randomly primed DNA probes using standard protocols (18). Probes were generated from purified PCR products used in arraying and subjected to cycle sequencing with T7 and T3 primers using recommended protocols and an ABI3700 automated sequencing apparatus.

RESULTS

Specificity of expression profiles. An important measure of any test's utility is its specificity. Specificity is a function of the rate of positive results in a defined population. Knowing the rate and factors that influence the proportion of false-positive results is essential for interpreting any microarray expression data. Unfortunately, the specificity of microarray expression analysis has not been studied rigorously, and there is little consensus on the number of replicates needed or on criteria used to identify differentially regulated genes. In lieu of documenting specificity, investigators typically set a "cutoff," or threshold, criterion above or below which changes in expression are deemed meaningful. This approach necessitates performance of second confirmatory tests, by either Northern or quantitative PCR analysis. Such confirmation, however, usually involves only a subset of the genes whose expression is believed to be increased or decreased. In addition, specificity is unlikely to be uniform for all microarray expression experiments and is almost certain to vary as a function of differences in array production and in biologic systems.

To assess the specificity of our arrays and protocols in

the study of liver expression, we measured the false-positive rate as a function of threshold criteria and experimental repetition. We produced a set of arrays (M1.6K) that represented >1,600 murine genes. The cDNAs were taken randomly from a commercially obtained, sequence-verified, Unigene plasmid library. No effort was made to enrich the pool of cDNAs for liver-specific transcripts or for genes known or suspected to be involved in energy homeostasis. One microgram of polyA RNA was prepared from the liver of an 8-week-old C57BL/6J female mouse. The sample was divided equally; one half was used to generate Cy3-labeled cDNA, the other half was used to generate Cy5-labeled cDNA. The two differentially labeled samples were hybridized simultaneously to a M1.6K array, washed, and scanned with a fluorescent scanning confocal microscope. Using different lasers to excite each of the dyes, we obtained raw fluorescence data for each spot on the array. The pixels (10 μm × 10 μm) that define each spot were determined in a two-step process. Initially, a map that contained the location and size of each spot of DNA was loaded into an analysis program (Imagene, BioDiscovery) and subsequently confirmed visually by us. Thus, for each spot, total, median, mean, and background pixel fluorescent intensity were obtained for the two identical samples that had been labeled with two different fluorophores. From these intensity data, a ratio that represented the relative signal from each dye was calculated for every spot. Each ratio was multiplied by a normalization factor intended to account for systematic bias in the incorporation or scanning of the fluorescent dyes. For these calculations, any ratio <1 was transformed by taking its multiplicative inverse.

If the specificity of the expression arrays were ideal, then the normalized fluorescence ratio calculated for each spot in this experiment would equal 1. Any variation from unity, therefore, represents the technical specificity limits of our arrays and protocols. The distribution of ratios from this experiment was very tight (Fig. 1*A*). Fewer than 0.5% of spots had a normalized fluorescence ratio of >2. Thus, for a threshold of 2, a value used by many workers, the specificity of our array and protocol is >99%. As would be expected, the specificity of microarray expression analysis is a function of threshold ratio—as the threshold is increased, the specificity increases with a concomitant decrease in sensitivity. However, independently repeating the experiment and combining data across array experiments also would be expected to increase the specificity but with less reduction in sensitivity. Repeating this experiment two more times, with polyA RNA prepared from a second and third liver, demonstrated a similar distribution of ratios for each individual array (data not shown). Averaging the expression ratios across all three arrays, while keeping the threshold ratio at 2, increases the specificity to 99.9% (Fig. 1*B*). If there is no gene-specific bias or systematic error, then another and more conservative method for increasing specificity is to set a minimum ratio threshold across a set of arrays. In our test arrays, for example, there were no spots for which the expression ratio was 1.2 or greater on each of the three microarrays (Fig. 1*B*). Thus, if we set a minimum expression ratio threshold of 1.3, then we will detect differences with a specificity of >99.9%. Or, stated more directly, a difference

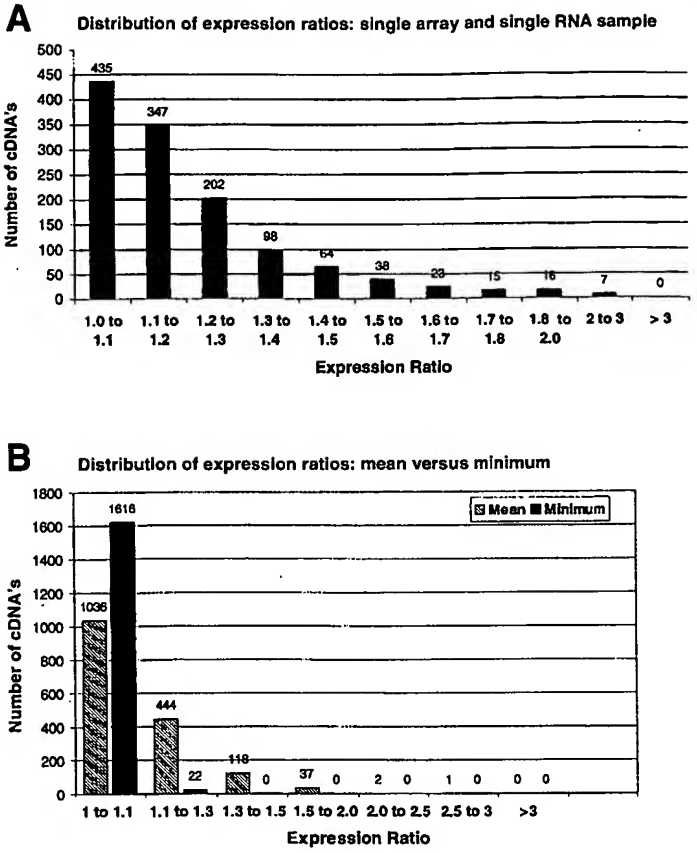

FIG. 1. Distribution of normalized expression ratios from microarray expression analysis. *A*: A polyA RNA sample was prepared from a single C57BL/6J liver, and 0.5-μg aliquots were converted separately to fluorescently labeled cDNA tagged with either Cy3 or Cy5. The fluorescent cDNAs were combined and hybridized to a single spotted microarray containing 1,638 murine cDNAs. Normalized ratios (see text for calculation of normalized expression ratios) were distributed over a range from 1–3. Totals do not equal 1,638 because not all genes represented on the array had detectable expression. *B*: PolyA RNA was isolated from three different C57BL/6J mouse livers and converted to fluorescently labeled cDNA using Cy3 and Cy5 dUTP. cDNAs derived from the same liver were hybridized to a single microarray, and normalized ratios were calculated for each gene on the microarray. A minimum ratio (■) and mean ratio (▨) were calculated for each cDNA and plotted as a distribution.

in expression of 30% or more across three microarrays will identify genes with true differences in expression, with a false-positive rate of <0.1%.

Gene expression profiles from livers of obese mice. To interrogate the gene expression profile from livers of wild-type and obese mice, we produced microarrays that contained 5,182 unique cDNAs (M5KA). So that we could monitor the internal reproducibility of our expression analysis, we spotted 384 genes in duplicate in the initial set of microarrays used in these studies. Livers were obtained from three 8-week-old female Lep^{ob}/Lep^{ob} mice and paired wild-type (Lep^{+}/Lep^{+}) control mice. PolyA RNA was extracted from each liver and used separately to generate fluorescently labeled cDNA. For one pair of RNA samples, Cy5 and Cy3 were switched so that on one array the Lep^{ob}/Lep^{ob} cDNA was labeled with Cy5, whereas on the other two it was labeled with Cy3. This was done to overcome any gene-specific dye bias. Differentially labeled cDNA from single control and single Lep^{ob}/Lep^{ob} mice were hybridized to a microarray. After washing and scanning, normalized expression ratios for each gene were

calculated. Guided by our specificity calculations, we selected the subset of genes that demonstrated a consistent 30% alteration across all three microarray experiments. Among this group were 91 genes that were consistently upregulated and 71 that were consistently downregulated in livers of obese mice (Table 1). For several of the genes, our data confirmed previous reports of altered expression in livers of obese mice, e.g., fatty acid synthase and insulin binding proteins. However, the majority of the genes were not known previously to be differentially regulated in Lep^{ob}/Lep^{ob} mice.

We calculated confidence intervals for the normalized expression ratio of each gene ($\alpha = 0.05$). Among the 162 genes that were consistently changed by 30% or more, only six have confidence intervals that cross unity, providing support that our expression profiling is robust and that our method identifies genes whose expression is significantly altered. To provide further confidence that the changes measured by our microarray expression experiments accurately reflected gene expression patterns within the livers of obese mice, we selected seven genes, six of which were differentially expressed on our microarrays, and performed Northern analysis. The RNA used for the Northern analysis was extracted from different mice than those used in the microarray expression studies but showed the same patterns of altered expression (Fig. 2). In addition, we resequenced these seven clones from our microarrays to confirm their identity.

The set of genes with altered expression within the livers of obese mice represent a broad spectrum of functional classes. The majority of the genes with altered expression are expressed sequence tags about which only sequence information is known. However, among the rest, we could identify nine intracellular signaling molecules, five cytochrome genes, three genes involved in glutathione metabolism, eight secreted proteins, and five transcription factors. The largest group of genes participate in intermediary metabolism, in particular fatty acid metabolism. (Striking is that the microarrays detect increases in expression of genes required for substrates necessary for fatty acid synthesis and fatty acid synthesis [fatty acid synthase, pyruvate dehydrogenase E1-α subunit, fructose bisphosphate aldolase] as well as increases in expression of genes involved in fatty acid oxidation [sterol carrier protein 2, hydroxymethylglutaryl (HMG)-CoA synthase 2, HMG-CoA lyase, 3-hydroxyacyl-CoA dehydrogenase, methylmalonyl-CoA mutase, medium chain acyl-CoA dehydrogenase].) On our array, no genes that are known to be involved in fatty acid metabolism were suppressed in livers of obese mice. These data suggest that in obese mice, leptin deficiency upregulates the transcription—or suppresses the mRNA turnover—of genes that regulate both fatty acid synthesis and oxidation. The transcription factors peroxisomal proliferating activated receptor-α (19) and sterol regulatory element-binding protein 1 (SREBP-1, also known as ADD1) (20) positively regulate the expression of genes involved in the oxidation and synthesis of fatty acids, respectively. The expression of both are elevated in Lep^{ob}/Lep^{ob} mice and thereby provide a transcriptional mechanism for the upregulation of a broad spectrum of fatty acid metabolism genes.

In human obesity, there is an increased risk of cardiovascular disease and mortality (21,22). However, Lep^{ob}/Lep^{ob} are relatively resistant to atherogenesis and have elevated concentrations of serum HDL particles (23,24). The basis of these two subphenotypes remains poorly understood. The elevation of HDL and its reduction by low doses of leptin result from altered hepatic metabolism of HDL (25). Among the genes altered in livers Lep^{ob}/Lep^{ob} mice, we identified four with potential roles in modulating serum cholesterol and thereby atherogenic susceptibility: HDL-binding protein, HMG-CoA synthase 2, HMG-CoA lyase, and apolipoprotein A-IV (Apo A-IV). All show increased expression in livers of obese mice. If the increased expression of any of these four genes mediates the atherogenic-resistant and elevated HDL subphenotype in obese mice, then their expression would be expected to normalize with short-term leptin treatment as HDL levels decrease.

Leptin-regulated gene expression in livers of obese mice. Treating Lep^{ob}/Lep^{ob} mice with leptin rapidly reverses many aspects of the obese phenotype (26). Within 1 h of peripheral or intracerebroventricular leptin administration, serum glucose and insulin decline. Within 24 h, food intake decreases more than 50%, and within 3 days, total body weight is significantly lower. To identify genes whose expression is modulated by leptin, we compared the expression profiles of obese mice that were treated for 1 week with leptin with those treated with vehicle. After 1 week of daily intraperitoneal leptin treatment, obese mice lost 17% of their body mass, whereas the vehicle-treated obese mice continued to gain weight (4%). As expected, Lep^{ob}/Lep^{ob} mice that were pair-fed with leptin-treated mice lost less body mass (13%) than leptin-treated mice (17%). Liver RNA was extracted from three mice in each treatment group and converted individually to fluorescently labeled cDNA. cDNA from each leptin-treated mouse was paired with a vehicle-treated or pair-fed cDNA sample and hybridized to a microarray. Data from the six microarrays identified sets of genes whose expression was altered between obese mice and obese mice that were treated with leptin and between obese mice that were treated with leptin and pair-fed obese mice.

The profiles generated from arrays that compared leptin- and vehicle-treated mice identified 29 genes whose expression was consistently altered 30% or more (Table 2); the comparison of leptin-treated and pair-fed profiles consistently detected such differences in expression of 35 genes (Table 2). Of the 158 genes whose expression differed between livers of obese and wild-type type, 11 showed correction or partial correction with short-term leptin treatment. The number of genes with corrected expression is a small fraction of the total and likely reflects several inadequacies of short-term treatment in mice with long-term leptin deficiency. Specifically, long-term deficiency is likely to have developmental consequences that are not correctable with 1 week of leptin treatment. In addition, once-daily intraperitoneal injections do not mimic the complex responses of ambient leptin to feeding and circadian rhythms (27,28). Finally, the very stringent criteria that we set for identifying differentially expressed genes likely will overlook genes whose expression is only partially corrected by leptin.

Taken together, the expression data from all experi-

TABLE 1
Regulation gene expression in leptin-deficient mice

Unigene cluster	Functions	Gene name	Expression ratio ob/ob:wild-type
Upregulated in ob/ob mice			
Mm.4533	Lipoprotein metabolism	Apo A-IV	10.3 (6.1–14.5)
Mm.20697		EST	7.3 (4.0–10.6)
Mm.23223		EST	5.4 (−0.9–11.7)
Mm.1620	Phospholipid binding protein	Annexin V	4.0 (3.1–4.9)
Mm.27136		EST highly similar to IIP2	3.7 (2.5–4.9)
Mm.22966		EST	3.6 (0.6–6.6)
Mm.31741		EST moderately similar to glyceraldehyde 3-phosphate dehydrogenase	3.3 (2.8–3.8)
Mm.23601		EST	3.2 (0.9–5.6)
Mm.3444	Signaling molecule	Bromodomain-containing 2	3.1 (2.7–3.4)
Mm.32353		EST	3.0 (0.7–5.4)
Mm.1620	*Phospholipid binding protein*	*Annexin V*	3.0 (2.8–3.2)
Mm.14022	Respiratory chain	Cytochrome C oxidase, subunit VIIIa	3.0 (1.4–4.5)
Mm.30567		EST	2.5 (2.0–3.0)
Mm.20407		EST weakly similar to pancreatic lipase–related protein 1	2.5 (1.6–3.3)
Mm.7524	Cytoskeletal protein	Dystroglycan 1	2.5 (1.2–3.7)
Mm.300		Carbonic anhydrase 3	2.4 (2.1–2.6)
Mm.3760	Fatty acid metabolism	Fatty acid synthase	2.4 (1.1–3.8)
Mm.27395		EST weakly similar to glutathione S-transferase	2.3 (1.6–3.1)
Mm.43415	Respiratory chain	Cytochrome C oxidase, subunit VI a, polypeptide 1	2.3 (1.4–3.2)
Mm.1779	Fatty acid metabolism	Sterol carrier protein 2	2.3 (2.1–2.4)
Mm.28882		EST moderately similar to elongation factor 1-α	2.3 (1.8–2.7)
Mm.2329		Hemoglobin β-pseudogene bh3	2.2 (1.4–3.0)
Mm.21571		EST	2.2 (1.6–2.7)
Mm.14796	Oxidative stress	Glutathione S-transferase	2.1 (1.6–2.6)
Mm.2475		Glutaryl-CoA dehydrogenase	2.1 (1.4–2.8)
Mm.16763	Glycolysis	Fructose-bisphosphate aldolase A	2.1 (1.4–2.8)
Mm.2607	Stress protein	Heat-responsive protein-12	2.1 (2.0–2.2)
Mm.32261		EST	2.1 (1.3–2.8)
Mm.10633	Ketone body production	HMG-CoA synthase 2	2.0 (1.8–2.3)
Mm.2607	*Stress protein*	*Heat-responsive protein-12*	2.0 (1.9–2.2)
Mm.1703	Cytoskeletal protein	Tubulin-β 5	2.0 (1.8–2.2)
Mm.1155	Cytoskeletal protein	Tubulin-α 4 chain	2.0 (1.5–2.5)
Mm.32254		EST	2.0 (1.0–3.0)
Mm.29057		EST highly similar to myosin regulatory light chain 2-A	2.0 (1.5–2.5)
Mm.8911	Protein processing	Proteasome-β-type subunit 5	2.0 (1.5–2.4)
Mm.22941		EST	2.0 (1.0–2.9)
Mm.2648	Signaling molecule	Calmodulin	1.9 (1.8–2.1)
Mm.2491	Fatty acid metabolism	3-Hydroxyacyl-CoA dehydrogenase	1.9 (1.3–2.5)
Mm.5121	Peptide metabolism	Peptidylglycine α-amidating monooxygenase	1.9 (1.2–2.7)
Mm.19094	*Respiratory chain*	*Cytochrome C oxidase, subunit VI a, polypeptide 1*	1.9 (1.4–2.4)
Mm.27677	Respiratory chain	EST highly similar to NADH-ubiquinone oxidoreductase 13Kd-b subunit	1.9 (1.5–2.3)
Mm.28683		EST	1.9 (1.4–2.3)
Mm.12556	*Mitochondrial metabolism related*	*Uncoupling protein 2*	1.8 (0.8–2.9)
Mm.1025	Transcription factor	NFED 2	1.8 (1.4–2.3)
Mm.29240		EST	1.8 (1.0–2.7)
Mm.3164	Respiratory chain	Cytochrome P450 2D9	1.8 (1.6–2.1)
Mm.7500		Ferritin light chain 1	1.8 (1.4–2.2)
Mm.30250		EST highly similar to antiquitin	1.8 (1.7–1.9)
Mm.29842	Respiratory chain	EST highly similar to NADH-ubiquinone oxidoreductase 51Kd subunit	1.8 (1.6–2.0)
Mm.23448		EST	1.8 (1.6–2.0)
Mm.3476	*Signaling molecule*	Catenin-β	1.8 (1.1–2.5)
Mm.9703	Copper transport protein	Atox1	1.8 (1.3–2.2)
Mm.31571		EST	1.8 (1.6–2.0)
Mm.2585		Brain protein I3	1.8 (1.1–2.4)
Mm.37656		EST moderately similar to proteasome Z subunit precursor	1.8 (1.5–2.0)

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TABLE 1
Continued

Unigene cluster	Functions	Gene name	Expression ratio ob/ob:wild-type
Mm.35081		EST weakly similar to titin	1.7 (1.2–2.3)
Mm.424	Respiratory chain	ATPase, Na+/K+ β_3 polypeptide	1.7 (1.4–2.1)
Mm.22668	Ketone body production	HMG-CoA lyase (mitochondrial)	1.7 (1.3–2.1)
Mm.32259		EST	1.7 (1.3–2.1)
Mm.26993	Respiratory chain	Cytochrome P450 3A25	1.7 (1.4–2.0)
Mm.30806	Protein synthesis	60S Ribosomal protein L19	1.7 (1.4–2.0)
Mm.24314		EST	1.7 (1.5–1.8)
Mm.31336		EST	1.7 (1.4–1.9)
Mm.4266		Integral membrane protein 2 B	1.6 (1.5–1.8)
Mm.37097		EST	1.6 (1.2–2.1)
Mm.29821		Peroxiredoxin 3	1.6 (1.4–1.9)
Mm.29628	Oxidative stress	EST weakly similar to acetylglucosaminyltransferase-like protein	1.6 (1.3–1.9)
Mm.26949		EST highly similar to electron transfer flavoprotein α-subunit	1.6 (1.3–1.9)
Mm.2576		BP 44-like Protein	1.6 (1.2–2.0)
Mm.13886	Helicase	*Sui1*	1.6 (1.3–1.9)
Mm.1970	Carbohydrate metabolism/ signaling protein	Protein phosphatase 1, catalytic subunit, α isoform	1.6 (1.2–2.0)
Mm.34470		EST	1.6 (1.2–2.0)
Mm.32251		EST	1.6 (1.3–1.9)
Mm.29633		EST	1.6 (1.3–1.8)
Mm.13886		*Sui1*	1.6 (1.4–1.7)
Mm.34775	Carbohydrate metabolism	Pyruvate dehydrogenase Elα subunit	1.6 (1.4–1.8)
Mm.426	Glutathione metabolism	Glutathione S-transferase, pi 2	1.6 (1.3–1.8)
Mm.22485		EST weakly similar yeast D-lactate dehydrogenase	1.5 (1.4–1.7)
Mm.2050		EST highly similar to ribosomal protein L15	1.5 (1.3–1.7)
Mm.26272		EST	1.5 (1.3–1.8)
Mm.4859		EST highly similar to Rer1 protein	1.5 (1.4–1.7)
Mm.29677		EST	1.5 (1.4–1.7)
Mm.2491	Fatty acid metabolism	*3-hydroxyacyl CoA dehydrogenase*	1.5 (1.2–1.8)
Mm.30012	Lipoprotein metabolism	HDL-binding protein	1.5 (1.4–1.6)
Mm.9925	Carbohydrate metabolism	NADP-dependent isocitrate dehydrogenase	1.5 (1.4–1.6)
Mm.32222		EST	1.5 (1.4–1.6)
Mm.42472	Microsomal modifying enzyme	UDP-glucuronosyltransferase 1 family member 1	1.5 (1.4–1.6)
Mm.4299	Fatty acid metabolism	Methylmalonyl CoA mutase	1.5 (1.3–1.6)
Mm.1486	ADP ribosylation factor 4	ADP ribosylation factor 4	1.4 (1.4–1.5)
Mm.46028		EST highly similar to CGI-26 protein	1.4 (1.4–1.4)
Mm.21209		EST	1.4 (1.3–1.5)
Mm.28492		EST highly similar to KIAA0652 protein	1.4 (1.3–1.5)
Mm.23165		EST weakly similar to ankyrin repeat-containing protein	1.4 (1.3–1.5)
Downregulated in ob/ob mice			
Mm.29973	Respiratory Chain	Cytochrome P450, 2c40	0.30 (0.25–0.35)
Mm.141936	Serum Protein	IGF-binding protein 2	0.35 (0.28–0.42)
Mm.32801		EST	0.47 (0.40–0.55)
Mm.14455	Signaling protein	Transforming growth factor, β induced, 68 kDa	0.54 (0.48–0.59)
Mm.2632	Transcription factor	Latexin	0.55 (0.47–0.62)
Mm.3288	Transcription factor	T-lymphocyte regulatory protein 1	0.55 (0.44–0.66)
Mm.28502	RNA metabolism	Double-stranded RNA-specific adenosine deaminase	0.56 (0.30–0.81)
Mm.32824		EST	0.57 (0.41–0.73)
Mm.955	Protein trafficking	μ-chain association protein	0.58 (0.45–0.70)
Mm.32225		EST	0.58 (0.41–0.75)
Mm.29014	Signaling protein	RAS-related C3 botulinum substrate 1	0.58 (0.38–0.78)
Mm.30694		EST	0.58 (0.39–0.78)
Mm.22173	Cell adhesion	Selectin, platelet (p-selectin) ligand	0.59 (0.53–0.65)
Mm.19131	Immune response	Complement C3	0.60 (0.50–0.69)
Mm.142	*Signaling protein*	*Lymphocyte protein tyrosine kinase*	0.60 (0.37–0.83)
Mm.4559	Glutathione metabolism	γ-glutamyl transpeptidase	0.61 (0.45–0.77)
Mm.16716	Lysosomal protein	Lysosomal membrane glycoprotein 1	0.61 (0.52–0.69)
Mm.3420	Transcription factor	NF-κB1	0.61 (0.48–0.75)

Continued on following page

TABLE 1
Continued

Unigene cluster	Functions	Gene name	Expression ratio ob/ob:wild-type
Mm.32829		EST	0.61 (0.46–0.77)
Mm.833	Protein trafficking	Clathrin coat assembly protein AP19	0.61 (0.48–0.75)
Mm.29524	Membrane channel	Nuclear chloride channel protein	0.61 (0.46–0.76)
Mm.23936		EST	0.61 (0.57–0.66)
Mm.30603		EST	0.63 (0.52–0.74)
Mm.13036		EST weakly similar to TSC501	0.63 (0.50–0.77)
Mm.6407		EST	0.63 (0.49–0.78)
Mm.24263		EST	0.64 (0.56–0.71)
Mm.542	RNA metabolism	RNA-binding motif protein 4	0.64 (0.57–0.71)
Mm.20874	Protein processing	Peptidylarginine deaminase type IV	0.64 (0.53–0.76)
Mm.20964	Signaling protein	Guanine nucleotide binding protein-α o	0.65 (0.60–0.70)
Mm.32274		EST	0.65 (0.54–0.76)
Mm.22963		EST highly similar to TEB4 protein	0.65 (0.59–0.72)
Mm.42321		EST	0.65 (0.56–0.74)
Mm.32266		EST	0.65 (0.53–0.78)
Mm.32472		EST	0.66 (0.61–0.71)
Mm.32447		EST	0.66 (0.52–0.79)
Mm.31837		EST	0.66 (0.58–0.74)
Mm.11946		EST weakly similar to a thyroid hormone responsive gene	0.66 (0.56–0.76)
Mm.23037		EST	0.66 (0.54–0.78)
Mm.22540		EST	0.67 (0.62–0.70)
Mm.13835		EST weakly similar to p190-B	0.67 (0.56–0.77)
Mm.32303		EST	0.67 (0.51–0.82)
Mm.32344		EST	0.67 (0.66–0.69)
Mm.2453	Hormone	Somatostatin	0.67 (0.63–0.72)
Mm.24004		EST	0.67 (0.59–0.76)
Mm.34298		EST	0.68 (0.62–0.73)
Mm.21049	Amino acid metabolism	Lysyl oxidase-2	0.68 (0.56–0.80)
Mm.32795		EST	0.69 (0.67–0.69)
Mm.28889		EST	0.69 (0.65–0.72)
Mm.35774		EST weakly similar to MAP kinase phosphatase-1	0.69 (0.63–0.75)
Mm.27573		EST	0.69 (0.57–0.81)
Mm.35134	Calcium transport	Ca++ transporting ATPase	0.69 (0.57–0.81)
Mm.142	Signaling protein	Lymphocyte protein tyrosine kinase	0.69 (0.63–0.76)
Mm.3130	Cytoskeletal protein	Kinesin heavy chain member 4	0.69 (0.64–0.75)
Mm.23038		EST	0.70 (0.65–0.74)
Mm.29381		EST weakly similar to centromeric protein E	0.70 (0.66–0.74)
Mm.32121		EST	0.70 (0.68–0.73)
Mm.32421		EST	0.71 (0.65–0.76)
Mm.30743		EST	0.71 (0.68–0.74)
Mm.32440		EST	0.71 (0.69–0.74)
Mm.42704		EST	0.71 (0.67–0.76)
Mm.15105	Signaling protein	Inositol polyphosphate-5-phosphatase, 145 kDa	0.72 (0.68–0.75)
Mm.54517		EST highly similar to nuclear pore complex protein NUP155.	0.72 (0.66–0.78)
Mm.45985	Metalloprotease	Bone morphogenetic protein 1	0.72 (0.67–0.77)
Mm.23007		EST	0.72 (0.72–0.73)
Mm.1685	Signaling protein	Plexin 3	0.72 (0.69–0.76)
Mm.6977	Protein processing	Trypsin 4	0.72 (0.68–0.77)
Mm.32515		EST	0.73 (0.70–0.76)
Mm.22789		EST weakly similar to retinal-cadherin	0.73 (0.73–0.74)
Mm.28854		EST weakly similar to type VI collagen-α 3 subunit	0.74 (0.72–0.77)
Mm.22818		EST	0.75 (0.73–0.76)
Mm.32162		EST	0.75 (0.73–0.77)

EST, expressed sequence tag.

ments can be used to identify seven groups of genes whose hepatic expression is modulated directly or indirectly by leptin (Table 3). The largest group (D) contains 146 genes that are only differentially expressed between livers of Lep^{ob}/Lep^{ob} and wild-type mice. These are genes whose expression is altered by long-term leptin deficiency and not restored by short-term treatment or caloric restriction. The group with the fewest regulated genes contains three

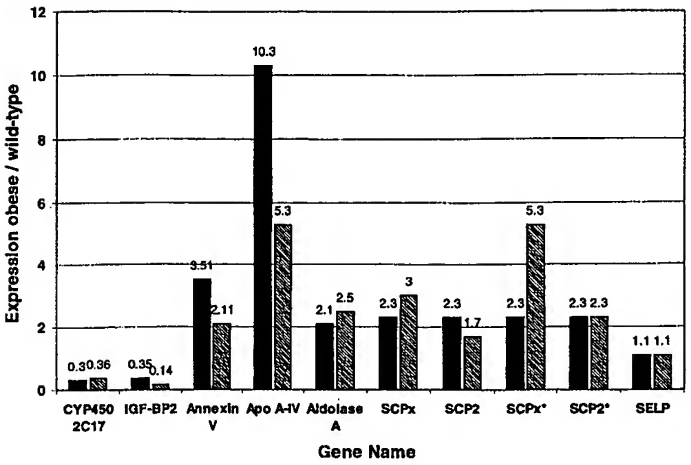

FIG. 2. Relative expression of select transcripts from the livers of obese and wild-type C57BL/6J mice. Total RNA was extracted from the livers of mutant obese and wild-type mice and electrophoresed through 1.2% agarose/MOPS gels. The RNA was blotted onto nylon membranes and probed with ^{32}P-labeled PCR fragments to seven genes. Ratios from Northern analyses were derived from phosphoimager intensities after normalization with 28S ribosomal RNA. Microarray ratios are the mean ratio from three separate microarray experiments. CYP 450 2C17, cytochrome P450 subunit 2C17; IGF-BP2, insulin-like growth factor–binding protein-2; aldolase A, fructose 2,6 bisphosphate aldolase A; SCPx/SCP2, sterol carrier protein x/2; SELP, selenoprotein. The sterol carrier protein x/2 gene generates four transcripts, two of which (SCPx and SCPx*) code for isoforms with thiolase activity and two of which (SCP2 and SCP2*) do not.

transcripts that are specifically regulated by short-term effects of leptin. In the absence of leptin and independent of energy balance, these genes are downregulated. (Hence, in comparison with leptin-treated or wild-type mice, leptin-deficient obese, mice whether gaining weight (fed ad libitum) or losing weight (pair-fed), express lower levels of these three genes.) For two of the leptin-dependent transcripts, there is little functional information, only sequence similarity data. One is similar to the glycolytic enzyme glyceraldehyde 3-phosphate dehydrogenase, and the other is highly similar to an insulin-regulated transcript. The third leptin-dependent transcript encodes Apo A-IV, a gene implicated in cholesterol trafficking, feeding behavior, and resistance to atherosclerosis.

Another small group of genes (B) is altered in the livers of *Lep^{ob}/Lep^{ob}* mice and partially corrected by leptin treatment and pair feeding. Hence, the dysregulation of these genes is reversible by decreased food intake or negative energy balance and is not dependent on leptin per se. They likely are part of pathways that mediate signals and responses to negative energy balance, e.g., increased fatty acid metabolism, and are not likely to mediate the increased metabolic efficiency, insulin resistance, or atherosclerosis resistance of the obese phenotype.

Although hyperphagic and obese, leptin-deficient *Lep^{ob}/Lep^{ob}* mice are postulated to exist in a state of perceived starvation (29). Thus, with free access to food, they behave as though they have been calorie-restricted, eating voraciously. However, their hyperphagic response can be enhanced further with fasting (30). This suggests that there is a set of nutrient-sensing pathways that are activated further in the fasted or calorie-restricted obese mice (31). The set of genes (C) that increased in expression in obese and pair-fed obese mice, which are not correctable with short-term leptin treatment, may represent genes that are part of the augmentable hyperphagic response of *Lep^{ob}/Lep^{ob}* mice.

The phenotype of calorie-restricted *Lep^{ob}/Lep^{ob}* mice has not been explored fully, but in addition to an augmented hyperphagic response, obese mice survive longer than wild-type mice under severe calorie restriction (32). The subset of genes (G)—only induced or repressed in calorie-restricted obese mice when compared with leptin-treated mice—are candidates for genes in metabolic and signaling pathways that mediate the increased survival phenotype of obese mice. Finally, the direct comparison of expression profiles between mice that gained (obese vehicle) and lost weight (obese leptin) detected 18 genes with consistently altered expression. The expression of these genes (F) is independent of leptin and inversely regulated in negative and positive energy balance states.

DISCUSSION

The emergence of new technologies to analyze variations of sequence and expression of genes provides the tools to identify the complex interactions that underlie the physiology of both normal and pathologic states. In the studies reported here, spotted cDNA microarrays were produced and used to monitor the gene expression patterns from wild-type, leptin-deficient *Lep^{ob}/Lep^{ob}* mice, and *Lep^{ob}/Lep^{ob}* mice that were treated with leptin and calorie restriction. Differences in expression were measured with high specificity (>99.9%). The assessment of sensitivity requires a standard that identifies all true positives and all false negatives. Unfortunately, there is no simple way to identify these data with an expression array. However, Pollack et al. (33) provided an estimate of spotted array sensitivity. Using single spotted cDNA microarrays probed with labeled genomic DNA, they detected differences in copy number with a sensitivity of 55% when a threshold ratio of 1.5 was used. That is, genes for which there was a known two-fold difference in copy number were detected 55% of the time when spots with normalized fluorescence ratios of 1.5 or greater were included. Given these data, our identification of differentially regulated hepatic genes probably is conservative and the sensitivity of our arrays likely is much lower than our specificity. The sensitivity of these experiments could have been increased by using a mean threshold ratio rather than stipulating a minimum consistent change across all microarrays. As is often the case, increasing the sensitivity would have required decreasing the specificity. In the context of the studies reported here, smaller data sets with fewer false-positive data points were much more useful than larger data sets with larger numbers of false-positive data points.

Leptin plays a central role in the regulation of feeding behavior, basal metabolism, glucose utilization, insulin sensitivity, and energy expenditure and thus overall in the regulation of energy homeostasis. In addition, leptin can modulate fertility, lipoprotein metabolism, bone turnover, and immune responses (34). Using microarrays, we identified more than 160 genes whose expression is altered in the livers of leptin-deficient *Lep^{ob}/Lep^{ob}* mice. By further comparing expression among leptin-treated and calorie-restricted *Lep^{ob}/Lep^{ob}* mice, we correlated the expression of subsets of genes with known phenotypic responses to pharmacologic and dietary interventions. The goal of these

TABLE 2
Regulation of genes

Unigene cluster	Gene name	Expression ratio (ob/ob vehicle) (ob/ob leptin)	Expression ratio (ob/ob) (wild-type)
Regulated by leptin treatment in ob/ob mice			
Mm.20697	EST	7.3 (4.3–10.2)	7.44
Mm.23889	EST	4.2 (2.4–6.0)	No change
Mm.4533	Apol A-IV	3.9 (2.6–5.2)	10.3
Mm.16763	Fructose-bisphosphate aldolase a	3.1 (1.7–4.5)	2.34
Mm.31741	EST moderately similar to Glyceraldehyde 3-phosphate dehydrogenase	2.7 (1.7–3.8)	3.39
Mm.23223	EST	2.6 (1.7–3.4)	5.28
Mm.27136	EST highly similar to IIP2	2.3 (0.7–3.9)	3.59
Mm.41853	Deoxyribonuclease II	2.1 (1.4–2.9)	No change
Mm.18213	Tubulin 5 β	2.1 (1.6–2.7)	1.83
Mm.24148	EST	2.1 (1.5–2.7)	No change
Mm.1620	Annexin V	2.1 (1.4–2.8)	2.91
Mm.28100	L-specific multifunctional β-oxidation protein	2.0 (1.5–2.6)	2.09
Mm.24742	Pantothenate kinase 1-β	1.8 (1.2–2.4)	1.75
Mm.18549	EST weakly similar to testatin	1.7 (1.5–1.9)	No change
Mm.20288	EST highly similar to glutathione reductase	1.7 (1.4–2.0)	No change
Mm.20925	Guanine nucleotide regulatory protein	1.7 (1.4–2.0)	No change
Mm.140158	Cytochrome P450, 51	1.6 (1.2–2.0)	No change
Mm.42795	IL-4–derived transcript	1.5 (1.4–1.6)	No change
Mm.32101	EST	1.5 (1.3–1.6)	No change
Mm.11816	EST	1.4 (1.3–1.5)	1.56
Mm.8903	Branched chain-α ketoacid dehydrogenase kinase	1.4 (1.3–1.5)	No change
Mm.14099	Hematopoietic zinc finger protein	1.3 (1.3–1.4)	No change
Mm.23304	TMEFF2	0.75 (0.74–0.77)	No change
Mm.34527	EST	0.7 (0.7–0.8)	No change
Mm.27539	EST	0.7 (0.6–0.8)	No change
Mm.29068	EST	0.6 (0.5–0.8)	No change
Mm.23443	EST	0.6 (0.4–0.9)	No change
Mm.3040	EST	0.6 (0.5–0.7)	No change
Mm.23252	EST	0.5 (0.2–0.8)	No change
Regulated by leptin independent of food intake			
Mm.30479	EST	3.60 (1.38–5.83)	
Mm.23581	EST	3.57 (0–7.49)	
Mm.2041	Metallothionein 1	2.67 (0.03–5.31)	
Mm.4533	Apo A-IV	2.57 (1.51–3.63)	
Mm.34446	Cyclin-dependent kinase inhibitor 1A-p21	2.21 (1.76–2.67)	
Mm.2585	Brain protein I3	1.97 (1.69–2.24)	
Mm.27136	EST highly similar to IIP2	1.96 (1.14–2.78)	
Mm.27506	EST	1.91 (1.63–2.19)	
Mm.27138	EST	1.73 (0.82–2.64)	
Mm.23411	EST	1.72 (1.59–1.85)	
Mm.31741	EST moderately similar to glyceraldehyde 3-phosphate dehydrogenase	1.71 (1.23–2.18)	
Mm.29842	NADH dehydrogenase flavoprotein 1	1.70 (1.63–1.77)	
Mm.44501	EST highly similar to gastrula zinc finger protein	1.69 (1.14–2.25)	
Mm.5238	Guanine nucleotide-binding protein α-13 subunit	1.67 (0.58–2.77)	
Mm.26709	EST	1.67 (1.56–1.79)	
Mm.17533	EST highly similar to BAP2-β	1.62 (1.29–1.95)	
Mm.2605	Retino-binding protein 4	1.60 (1.46–1.73)	
Mm.10633	HMG-CoA	1.60 (1.49–1.71)	
Mm.34742	EST highly similar to citrin	1.56 (1.18–1.95)	
Mm.29451	EST weakly similar to B0414.8	1.55 (1.48–1.62)	
Mm.24014	EST	1.55 (1.17–1.93)	
Mm.29882	EST	1.54 (1.36–1.71)	
Mm.33729	EST	1.51 (1.20–1.83)	
Mm.28882	EST moderately similar to elongation factor 1-α	1.51 (1.36–1.66)	
Mm.4266	Integral membrane protein 2B	1.51 (1.23–1.79)	
Mm.20354	Kinesin light chain 1	1.47 (1.30–1.64)	
Mm.31810	EST	1.46 (1.22–1.71)	
Mm.21761	EST	1.46 (1.10–1.81)	
Mm.39099	EST highly similar to zinc finger protein 91	1.46 (1.41–1.50)	
Mm.22570	Histone H1.1	1.45 (1.39–1.52)	
Mm.29682	EST	1.43 (1.28–1.58)	
Mm.13886	Sui1	1.41 (1.38–1.43)	
Mm.23325	EST	1.39 (1.34–1.43)	
Mm.18443	GLUT2	1.37 (1.31–1.42)	
Mm.5750	EST	0.70 (0.64–0.76)	

TABLE 3
Expression ratio patterns

Group	ob/ob/ wild-type	ob/ob vehicle/ ob/ob leptin	ob/ob pain-fed/ ob/ob leptin	No. of genes
A	Change	Change	Change	3
B	Change	Change	—	8
C	Change	—	Change	5
D	Change	—	—	151
E	—	Change	Change	0
F	—	Change	—	18
G	—	—	Change	26

studies was to identify candidate genes that mediate specific metabolic subphenotypes of leptin deficiency.

Three genes specifically modulated by leptin (group A) were identified. In obese mice, these genes are upregulated and are corrected by short-term leptin treatment. In comparison with leptin treatment, however, the expression of these genes was not altered by calorie restriction. This result underscores the observation that leptin's peripheral effects are not mediated solely by leptin's ability to modulate feeding behavior. Indeed, most of leptin's pleiotropic effects, including those that regulate insulin resistance, basal energy metabolism, fertility, lipoprotein metabolism, and immune responses, must be mediated, at least in part, by pathways that are independent of food intake and energy balance. Of the genes that we identified that are dependent on leptin but not on nutrient intake, two have not yet been characterized but do share sequence similarity to other genes. The third is an apolipoprotein.

Normally, Apo A-IV is secreted primarily into the portal circulation from the small intestine and rises in response to high-fat meals (35). A lesser amount is produced by the liver. Apo A-IV has been postulated to modulate feeding behavior negatively (36), regulate HDL metabolism (37), and impart atherosclerosis resistance (38,39). Our microarray profiles detected a 10-fold increase in hepatic expression of Apo A-IV in Lep^{ob}/Lep^{ob} mice. This increase is partially normalized after a short course of daily leptin injections. C57BL/6J mice that overexpress Apo A-IV in the liver have elevated serum HDL and are resistant to developing atherosclerosis, even when fed an atherogenic diet or when made genetically deficient in Apo E (38,39). Conversely, mice homozygous for a null mutation in the Apo A-IV gene have lower serum HDL (37). These data make Apo A-IV an excellent candidate gene for mediating the elevated HDL and atherosclerosis-resistant phenotypes of obese mice. The role of Apo A-IV in mediating these phenotypes can be tested directly. If, in obese mice, Apo A-IV is necessary for the elevated HDL levels and resistance to atherogenesis, then mice that are homozygous for null mutations in leptin and Apo A-IV, $<Lep^{ob}/Lep^{ob}>;<ApoA4^{-}/ApoA4^{-}>$, will be more susceptible to atherosclerosis than the Lep^{ob}/Lep^{ob} mice. In addition, mice that carry the lethal yellow agouti allele are resistant to both exogenous leptin and atherogenesis (23), and we would predict that levels of liver expression of Apo A-IV will be elevated in $<A^{y}/a^{+}>$ animals.

Another of the leptin-regulated transcripts (Unigene cluster Mm.27136) is highly similar (94% nucleotide sequence identity) to a human gene, insulin-induced protein 2 (IIP2). IIP2 is expressed in regenerating livers (40) and is upregulated in hepatocytes treated with insulin (41). These studies show that in hyperinsulinemic obese mice, the IIP2-like transcript is upregulated approximately fourfold. Leptin treatment, which lowers the expression of this gene, also normalizes insulin sensitivity, resulting in decreases in both serum glucose and insulin concentrations. Calorie restriction of obese mice can improve hyperglycemia and modestly reduce insulin concentration, but such restriction does not reverse the underlying insulin resistance and does not normalize IIP2-like expression. Shimomura et al. (42) recently argued that in leptin-deficient states, insulin is unable to downregulate gluconeogenesis and glycogenolysis appropriately, whereas its ability to stimulate lipogenesis remains intact. Specifically, they suggest that within the liver of leptin-deficient mice, insulin signaling diverges along two paths, one that remains insulin-sensitive and stimulates excess fatty acid production and one that is resistant to insulin downregulation and leads to excessive glucose synthesis. If correct, the IIP2-like transcript is a candidate for mediating the insulin-sensitive responses in leptin-deficient mice. An initial test of this hypothesis would measure the transcriptional response of the IIP2-like gene in obese mice treated with insulin. If the IIP2-like gene is part of a pathway that remains insulin-sensitive in the leptin-deficient mice, then its expression should increase further in tandem with the transcription factor SREBP1-c, which remains insulin-responsive in obese mice.

In conclusion, the complex pathways that regulate short- and long-term energy homeostasis remain poorly defined. By defining transcriptional perturbations that occur in the livers of Lep^{ob}/Lep^{ob} mice, we identified pathways and candidate genes whose hepatic function is altered by leptin deficiency. Using microarrays, we demonstrated upregulation of genes required for lipid metabolism and identified candidate genes that may mediate the metabolic subphenotypes of resistance to atherosclerosis and insulin-responsive fatty acid overproduction. These results suggest that such studies could be expanded fruitfully to include a larger set of genes, to interrogate other energy-regulating tissues (hypothalami, adipose tissue, and skeletal muscle), and to include other murine models of obesity (Ay/a^{+}, Cpe^{fat}/Cpe^{fat}, diet-induced). Through performance of more comprehensive expression analyses, additional candidate genes that contribute to obesity-related phenotypes and participate in regulating feeding behavior, energy partitioning, and energy expenditure could be identified.

ACKNOWLEDGMENTS

Supported in part by National Institutes of Health Grants R01-DK-52431 and T32-DK-07559, an American Diabetes Association Training Grant, the Lucille P. Markey Foundation, and the Columbia Innovation Enterprise.

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